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Upregulation of miR-21 and pro-inflammatory cytokine genes *IL-6* and *TNF-α* in promoting a pro-tumorigenic microenvironment in canine mammary carcinomas

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ABSTRACT

This study evaluated the gene expression of the pro-inflammatory cytokines, interleukin-6 (*IL-6*) and tumor necrosis factor alpha (*TNF-α*) in canine mammary tumors (CMTs), and correlated them with gene expression of miRNAs expected to regulate the secretion of pro-inflammatory cytokines within the tumor microenvironment (TME). Furthermore, gene expression of cytokines and miRNAs involved in tumor cell proliferation and invasion (*i.e.* miR-21; miR-124; miR-145) were correlated with tumor proliferation index (Ki67 index) to determine the prognostic value in CMTs. Twenty-six canine mammary samples were used, including 22 CMTs and 4 control samples. MiR-21, *IL-6* and *TNF-α* were upregulated in mammary carcinomas compared with controls ($p < 0.05$). MiR-146b was downregulated in CMTs compared with control cases ($p < 0.05$). *IL-6* expression showed a significant positive correlation with miR-21 and a negative correlation with miR-146b; while, *TNF-α* gene expression was positively correlated with miR-21 and miR-145 in mammary carcinomas. In carcinomas, the Ki67 index correlated positively with gene expression of *IL-6* and miR-21 and negatively correlated with miR-145 and miR-146b. Specifically, gene expression of *IL-6* and miR-21 was positively correlated with ki67 index >33.3%, whereas, expression of miR-145 and miR-146b was negatively correlated with ki67 index <33.3%. Results reinforce the concept of interaction between tumor cells and inflammatory cells within the TME, with a central role of *IL-6* and *TNF-α*. Since the upregulation of miR-21 reflects the gene overexpression of interleukins and the high proliferation index of tumor cells, this miRNA may be considered a biomarker with prognostic value in CMTs.

1. Introduction

The tumor microenvironment (TME) represents a complex network of signaling molecules, exchanged between neoplastic cells, infiltrating immune cells, and resident stromal cells, that cooperate to support tumor growth, malignant cell phenotype and influence cancer treatment outcome (Balkwill et al., 2012; Joyce, 2005; Quail and Joyce, 2013). The key role of inflammatory cell infiltration in tumorigenesis is not a new insight and would act as a propellant for genetic alterations and as an enhancer of tumor cell invasion and metastatic dissemination (Balkwill and Mantovani, 2001; Le Bitoux and Stamenkovic, 2008; Pollard, 2004). Consequently, investigations on the relationship between inflammation and mammary cancer pathogenesis has attracted considerable interest in canine patients in recent years (Carvalho et al., 2014; Carvalho et al., 2011; De Souza et al., 2018; Kim et al., 2010; Raposo et al., 2015a).

Cancer cells commonly induce an intrinsic and chronic inflammatory reaction to stimulate a pro-tumorigenic microenvironment, and the ongoing crosstalk between neoplastic and immune cells results in immunosuppression, invasion and cancer progression (Balkwill et al., 2012; Landskron et al., 2014; Mantovani et al., 2008; Quail and Joyce, 2013). Pro-inflammatory TME is primarily driven by bone-marrow-derived cells, such as monocytes and macrophages and other cell

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types, including T lymphocytes, and is orchestrated by several cytokines and chemokines, such as IL-6 and TNF-α (Dethlefsen et al., 2013; Germano et al., 2008; Kim et al., 2010; Raposo et al., 2015b). The effects of inflammatory cells can result from direct interactions with neoplastic cells and through indirect effects on other resident stromal cells, particularly cancer-associated fibroblasts and endothelial cells, supporting many of the hallmarks of cancer (Balkwill et al., 2005; Condeelis and Pollard, 2006; Hanahan and Weinberg, 2011). Indeed, the inflammatory infiltrate in tumor sites promotes angiogenesis, metastatic dissemination, enhancing the invasive potential of tumor cells through the epithelial-to-mesenchymal transition, mediated by the production of tissue remodeling proteases, growth factors, cytokines and chemokines (Balkwill et al., 2005; Condeelis and Pollard, 2006; Raposo et al., 2015b; Soria et al., 2011). In particular, IL-6 is a cytokine with pleiotropic activity, and plays a key role in the acquired immune response by stimulating the production of antibodies and the development of effector T cells (Tanaka et al., 2014). Dysregulated IL-6 exerts various effects on lymphocytes frequently encountered in chronic inflammatory diseases and cancer, with a key pathogenetic role in various non-immune diseases (Tanaka et al., 2014). Furthermore, the inflammation-associated cytokine IL-6 may contribute to tumor growth and resistance to therapy by activating survival mechanisms (Zarogoulidis et al., 2013). In addition to IL-6, TNF-α is an important mediator of inflammation and immune function, and in the TME, TNF-α acts as a potent inducer of the pro-inflammatory cytokines IL-1 and IL-6 (Martins et al., 2016). Moreover, TNF-α represents a key chemical mediator that induces both neoplastic cell proliferation and necrosis (Le Bitoux and Stamenkovic, 2008; Soria et al., 2011; Tang et al., 2002).

MicroRNAs (miRNAs), small non-coding RNA molecules involved in cell-cell signaling and communication, have emerged as crucial endogenous regulators of gene expression in cancer (Kohlhapp et al., 2015). As essential mediators between cancer cells and other cellular components of the TME, miRNAs are considered to be central players in regulating multiple aspects of cancer biology (Anfossi et al., 2018; Kohlhapp et al., 2015). Within the TME, the altered composition of tumor cell-derived miRNAs mediates dysregulated signaling and miRNA-mediated crosstalk generates a niche that enhance tumor cell proliferation, antitumor immune response and metastasis (Anfossi et al., 2018). Of note, cancer cell-derived miRNAs can regulate immune cell functions and have been suggested as key modulators of the inflammatory cascade during cancer progression, offering an interesting link between inflammation and cancer (Bell and Taylor, 2017; Chakraborty et al., 2020). In particular, miR-21 promotes nuclear factor-κB (NF-κB)-mediated production of IL-6 and TNF-α through its binding to Toll-like receptor 7/8 (TLR7/8) on macrophages, leading to the enhancement of the pro-inflammatory response (Anfossi et al., 2018; Chakraborty et al., 2020). Conversely, miR-146b inhibits NF-κB-dependent production of IL-6 and IL-6/STAT3-driven tumor cells migration and invasion in breast cancer (Xiang et al., 2014). Furthermore, miR-21 serves as a regulator of apoptosis along with tumor cell proliferation and invasion and suggested as a potential marker to detect metastasis (Xu et al., 2015; Zhang et al., 2016;). Contrariwise, miR-124 and miR-145 are tumor suppressor genes, which inhibits cell proliferation and invasion in breast cancer, and are downregulated in numerous cancers, including triple-negative breast cancer (Sachdeva et al., 2009; Zheng et al., 2016).

Mammary cancer is a common malignancy in female dogs and the creation of an inflammatory microenvironment plays a well-known role in promoting tumor onset and progression, thus representing a biological predictor of malignant tumor cell phenotype (Carvalho et al., 2014; De Souza et al., 2018). In particular, within the TME, T lymphocytes, identified as tumor-infiltrating lymphocytes (TILs) play a crucial role in shaping the immune response against cancer, and large numbers of CD3+ and CD4+ T lymphocytes and tumor-infiltrating macrophages is associated with shorter overall survival time in dogs with mammary carcinomas (Bujak et al., 2020; Franzoni et al., 2019). TILs can produce both type 1 (*e.g.* TNF-α) and type 2 (*e.g.* IL-6) cytokines and can encourage tumor growth by promoting angiogenesis and lymphangiogenesis (Kim et al., 2010). However, microenvironmental factors in CMTs are poorly characterized and the role of miRNAs on interleukin-mediated inflammation and cancer cell proliferation is still underexplored.

Therefore, to broaden the knowledge on the epigenetic mechanisms involved in the CMTs, this study aimed to evaluate the gene expression of pro-inflammatory *IL-6* and *TNF-α* and correlate them with the expression of miRNAs predicted to regulate the secretion of pro-inflammatory cytokines (*i.e.* miR-21, miR-146b). Association between histological grading, stromal tumor-infiltrating lymphocytes (TILs) and intratumoral necrosis were assessed, known to have a prognostic value. Furthermore, the possible correlation between the gene expression of cytokines and/or miRNAs involved in cell proliferation (*i.e.* miR-21; miR-124; miR-145) and the tumor proliferation index (Ki67 index) was evaluated to determine the prognostic value of these miRNAs in mammary tumors.

2. Material and methods

2.1. Mammary samples and histopathology

Twenty-six formalin-fixed, paraffin-embedded (FFPE) canine mammary samples were used in this study, including mammary tumors ($n = 22$), and nonneoplastic mammary tissue samples ($n = 4$) used as control cases for immunohistochemical and molecular analyses. Tumors were selected among cases routinely referred for diagnostic purposes in the archive of the AniCura Veterinary Hospital "I Portoni Rossi" (Bologna, Italy). Three μm-thick mammary tissue sections stained with hematoxylin-eosin (HE) were used for histopathological examination. Canine mammary tumors were classified by a board-certified pathologist (B.B.) according to Zappulli et al. (2019) and histological grading was performed according to Peña et al. (2013).

2.2. Evaluation of tumor-infiltrating lymphocytes (TILs) and intratumoral necrosis

The presence of inflammatory infiltrate and tumor necrosis was evaluated semi-quantitively on HE-stained slides, and performed by two investigators (J.M.A.; B.B.) using a double-headed microscope simultaneously. Evaluation of tumor-infiltrating lymphocytes (TILs) was performed according to Salgado et al. (2015) and Muscatello et al. (2022). In particular, the infiltration of stromal TILs (lymphocytes and plasma cells) was assessed by evaluating the whole tumor section at 20× of magnification and expressed as the area (%) occupied by TILs on the total intratumoral stromal area. Three categories were considered for infiltrating stromal TILs: Low (0–10%); Intermediate (11–40%); High (41–90%) (Salgado et al., 2015). The presence of intratumoral necrosis was described using a 3-point scoring system, as follow: 1 (low) when necrosis was absent or minimal (<10%); 2 (moderate), when the area of necrosis was 10 to 50% of the tumor area; 3 (high), when the necrotic area occupied >50% of the tumor area.

2.3. Immunohistochemistry (IHC)

Three μm-thick sections of FFPE mammary samples were used to perform IHC, dewaxed in xylene and rehydrated in ethanol. Treatment was performed with 3% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity, and heat-induced antigen retrieval was performed using citrate buffer pH 6.0 at 750 W in microwave for four, 5-min cycles. After pre-incubation with blocking solution (BS) for 30 min at room temperature, tissue sections were incubated overnight at 4 °C with primary antibody (monoclonal mouse Ki67; clone MIB-1, Dako, Denmark; diluted 1:600). The binding sites were revealed by a secondary antibody, diluted 1:200 (goat anti-mouse polyclonal; Dako, Denmark). Detection was performed using a commercial avidin-biotin

peroxidase kit (VECTASTAIN ABC Kits) and 3′,3′ – Diaminobenzidine (DAB) was used for visualization (ACH500-IFU, ScyTek Laboratories). Slides were counterstained with Meyer's hematoxylin. Normal canine intestine was included as an internal positive control in each IHC reaction. The negative control was considered by omitting the primary antibody.

Ki67 immunolabeling of cells was expressed as the percentage of positive nuclei in 500 counted neoplastic cells (Ki67 index) (manual image analysis, Image J Software, National Institute of Health, Bethesda, Maryland, USA). A cutoff of 33.3% Ki67-positive neoplastic cells was considered to discriminate between high- and low-proliferating CMTs (Nguyen et al., 2018).

2.4. Molecular investigations

2.4.1. Total RNA purification

Two sections, 10–15 μm-thick, of a representative area of FFPE mammary tumors and nonneoplastic mammary tissues identified in the paraffin block, were collected in a 1.5 ml sterile tube and used for total RNA purification using miRNeasy® FFPE Kit (QIAGEN, Milan, Italy; cat. no. 217504) and Deparaffinization Solution (QIAGEN, Milan, Italy; cat. no. 19093). RNA concentration and purity were evaluated using the Nanodrop Spectrophotometer (NanoPhotometer N50, IMPLEN, USA) and further stored at −20 °C until molecular investigations. In particular, the RNA quality was verified by assessing the 260/280 nm and 260/230 nm absorbance ratios, and all samples had values between 1.8 and 2.

2.4.2. cDNA synthesis and RT-qPCR for miRNAs

The cDNA synthesis and RT-qPCR for miRNAs have been detailed in our previous publications (Abbate et al., 2023; Abbate et al., 2022). Briefly, the reverse transcription (RT) reaction was performed using the miRCURY® LNA RT Kit (QIAGEN, Milan, Italy; cat. no. 339340) in a final reaction volume of 10 μl containing: 2 μl of 5× miRCURY RT Reaction Buffer; 1 μl of 10× miRCURY RT Enzyme Mix; 0.5 μl UniSp6 RNA spike-in provided as an internal quality control of cDNA synthesis; 4.5 μl of RNase-free water; 20 ng of RNA in 2 μl final volume. The reaction tubes were incubated for 60 min at 42 °C and for 5 min at 95 °C.

RT-qPCR was performed in duplicate for each sample in a final reaction volume of 20 μl, using: 10 μl of 2× miRCURY® LNA SYBR Green PCR Kit (QIAGEN, cat. no. 339345–339,346); 2 μl of specific miRCURY® LNA miRNA PCR Assays (QIAGEN, Milan, Italy; Cat. no. 339306); 2 μl of RNase-free water; 6 μl of cDNA (diluted 1:60). The following miRNA PCR assays were used: hsa-miR-21-5p (assay ID – YP00204230); cbr-miR-124 (assay ID – YP02103368); has-miR-145-5p (assay ID – YP00204483); has-miR-146b-5p (assay ID – YP02119310). Assays used as endogenous controls for normalization included: hsa-miR-16-5p (assay ID – YP00205702); hsa-let-7a-5p (assay ID – YP00205727) (Bulkowska et al., 2017; Fish et al., 2018).

RT-qPCR was performed using a SYBR Green chemistry in a Rotor-Gene® Real-Time PCR system (QIAGEN), with the following cycling conditions: 95 °C for 2 min, 40 cycles of 95 °C for 10 s and 56 °C for 60 s. Single-peak melting curves were used to evaluate the specificity of the reaction. The miRNA expression levels are presented in terms of fold change normalized to endogenous controls using the formula $2^{-\Delta\Delta Cq}$.

2.4.3. cDNA synthesis and RT-qPCR for IL-6 and TNF-α mRNA

For *IL-6* and *TNF-α* genes, the cDNA was synthesized using the Sensiscript® Reverse Transcription Kit (QIAGEN, Milan, Italy; cat. no. 205211), providing the ribonuclease inhibitor (R1158–2.5KU; Sigma-Aldrich, Co. St Louis, MO, 210 USA) and the oligo-dT primer (Oligo (dT)8; Catalog No. BIO-38029; Meridian Bioscience; Germany).

The RT reaction was set up in a final reaction volume of 20 μl, using: 2 μl of 10× RT Reaction Buffer; 2 μl of dNTP Mix; 1 μl of Sensiscript Reverse Transcriptase; 1 μl of Oligo-dT primer (10 μM); 0.5 μl of RNase inhibitor (10 units/μl). Finally, after denaturation at 65 °C for 5 min, a total of 30 ng of RNA for each sample was added to each RT tube and diluted in RNase-free water. The reverse transcription reactions were incubated for 60 min at 37 °C, and the cDNA stored at −20 °C.

RT-qPCR for *IL-6* and *TNF-α* mRNAs was performed using GoTaq® qPCR Master Mix (Promega, Milan, Italy; cat. no. A6002). Ribosomal protein S19 (*RPS19*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT*) were selected as housekeeping genes for normalization (Brinkhof et al., 2006; Etschmann et al., 2006). The sequences of primers are listed in Table 1.

The RT-qPCR reaction was performed in duplicate for each sample in a final reaction volume of 20 μl, using: 10 μl of GoTaq® qPCR Master Mix; 1.5 μl of Forward Primer (10 μM/μl); 1.5 μl of Reverse Primer (10 μM/μl); 5 μl of Nuclease-free water; 2 μl of cDNA template (1:10 diluted). The reaction was performed using a Rotor-Gene® Real-Time PCR system (QIAGEN), with the following cycling conditions: 2 min at 95 °C, 40 cycles of 15 s at 95 °C and for 1 min at 60 °C. Gene expression is presented in terms of fold change normalized to the housekeeping genes using the formula $2^{-\Delta\Delta Cq}$.

2.5. Statistical analysis

All data were tested for normal distribution using the Kolmogorov-Smirnov test. Since the data were not normally distributed ($p < 0.05$), a nonparametric statistical analysis was applied. In particular, the Kruskal-Wallis test was performed to assess significant differences in *IL-6*, *TNF-α* and miRNAs (miR-21, miR-124, miR-145, miR-146b) gene expression among nonneoplastic mammary tissues (Control group, Group C), benign CMTs (Group B) and malignant tumors (Group M1–3), followed by Dunn's multiple comparison test. The Mann Whitney test was applied to evaluate significant differences in the Ki67 index between benign (Group B) and malignant CMTs of different grade of malignancy (Group M1–3). Furthermore, the Mann Whitney test was applied to evaluate the difference in *IL-6*, *TNF-α* and miRNA gene expression among CMTs based on the Ki67 index (Ki67 index >33.3%, high proliferative tumors; Ki67 index <33.3%, low proliferative tumors).

Spearman correlation coefficients were calculated to evaluate the possible correlation between gene expression levels of interleukins (*i.e. IL-6*, *TNF-α*) and miRNAs (*i.e.* miR-21, miR-124, miR145, miR146b) in both benign (Group B) and malignant tumors (Group M1–3). Furthermore, the possible correlation between the Ki67 index and the gene expression levels of *IL-6*, *TNF-α*, miR-21, miR-124, miR-145 and miR-146b was evaluated in benign (Group B) and malignant tumors (Group M1–3). Spearman's test was applied to evaluate whether interleukins and miRNA gene expression correlated with Ki67 index >33.3% and < 33.3%. A linear regression model ($y = a + bx$) was applied to determine the degree of correlation. *P* value <0.05 was considered statistically significant. The statistical analysis was performed using Prism Software v. 9.00 (GraphPad Software Ltd., USA, 2020).

3. Results

3.1. Study population and mammary samples – Inflammatory infiltrate

Canine mammary tumors ($n = 22$) were surgically removed from a cohort of 13 females, aged 10.0 ± 2.0 (mean ± SD) years, and included benign tumors ($n = 7$; Group B) and mammary carcinomas ($n = 15$; Group M). Benign tumors were removed from females aged 8.5 ± 3.5 years, while for mammary carcinomas dogs aged 10.4 ± 1.4 years. Nonneoplastic mammary samples included 3 normal mammary gland samples and 1 sample of lobular hyperplasia (Group C), removed from 4 females, aged 8.4 ± 1.5 years. No positive lymph nodes were detected in the six females from whom 10 of the 15 mammary carcinomas were sampled; while lymph nodes were not evaluated in 4 females from which the remaining 5 carcinomas were removed. No distant metastases were detected in canine patients prior to surgery. Size ranged for all tumors

Table 1
Sequences of primers used for RT-qPCR (Brinkhof et al., 2006; Etschmann et al., 2006; Tamura et al., 2014).

Gene	Primer sequence (5′- 3′)		GenBank Accession No.
	Forward	Reverse	
IL-6	TTAAGTACATCCTCGGCAAAATCT	CAGTGCCTCTTTGCTGTCTTCA	NM_001003301
TNF-α	TCTCGAACCCCAAGTGACAAG	CAACCCATCTGACGGCACTA	NM_001003244
RPS19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	XM_533657
HPRT	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT	NM_000194

Interleukin (IL)-6; Tumor Necrosis Factor (TNF)-α; RPS19, Ribosomal protein S19; HPRT, Hypoxanthine phosphoribosyltransferase 1.

from 0.2 to 4 cm in diameter, with benign CMTs ranging from 0.2 to 2 cm and carcinomas ranging from 0.5 to 5 cm in diameter.

Benign tumors were classified as simple adenomas ($n = 4$), complex adenomas ($n = 2$) and benign mixed mammary tumor ($n = 1$). Simple adenomas were further classified into tubular adenoma ($n = 1$), tubulopapillary adenoma ($n = 1$) and intraductal papillary adenomas ($n = 2$). Malignant CMTs (n = 15) were diagnosed as tubular carcinomas ($n = 2$), tubulopapillary carcinoma ($n = 1$), complex carcinomas ($n = 3$), intraductal papillary carcinomas ($n = 2$), mixed carcinomas ($n = 2$), solid carcinomas ($n = 4$) and inflammatory carcinoma ($n = 1$). Malignant tumors were grade 1 ($n = 8$), grade 2 ($n = 2$) and grade 3 ($n = 5$). Lymphovascular invasion was histologically observed in 2 carcinomas (Table 2).

Most mammary carcinomas ($n = 7$) showed low stromal infiltration of TILs (0–10%). An intermediate TILs infiltration was observed in 4 malignant CMTs, and a high TILs infiltration was observed in the remaining 4 carcinomas. Tumor necrosis was absent in benign tumors, while in carcinomas, necrosis was absent or minimal in 4 cases (<10%; score 1), ranged from 10 to 50% in most malignant CMTs ($n = 10$) (score 2), while necrosis occupied >50% of the tumor area in only 1 intraductal papillary carcinoma, grade 1 (score 3). Data regarding the estimation of stromal TILs infiltration and tumor necrosis are shown in Table 2.

Representative canine mammary carcinomas with different stromal infiltration of TILs are presented in Fig. 1.

3.2. Immunohistochemistry

Considering benign *vs.* malignant canine mammary tumors, the Ki67 positivity was 21.97 ± 10.21% (mean ± SD) and 47.34 ± 22.79% (mean ± SD), respectively, with no statistically significant difference between benign and malignant tumors ($p > 0.05$). CMTs were distinguished according to the proliferation index in low proliferative tumor (Ki67 index <33.3%) ($n = 10$; 19.39 ± 7.93) and high proliferative tumors (Ki67 index >33.3%) ($n = 12$; 60.43 ± 13.86).

3.3. miRNAs gene expression in mammary samples

Statistical analysis of the data showed a significant effect of the group on the gene expression of miR-21 and miR-146b ($p < 0.05$); in contrast, no significant differences in miR-124 and miR-145 expression levels were found between groups ($p > 0.05$). In particular, a higher expression of the miR-21 gene was observed in benign and malignant CMTs compared with non-neoplastic mammary samples. Conversely, an opposite trend of expression was observed for miR-146b, showing lower expression levels in both groups B and M1–3 compared with control cases ($p < 0.05$) (Fig. 2) (Abbate et al., 2022).

3.4. IL-6 and TNF-α gene expression in mammary samples

A statistically significant effect of the group on *IL-6* and *TNF-α* gene expression was observed. In particular, higher expression of *IL-6* gene was observed in Group M1–3 compared with Groups C and B ($p < 0.05$), and higher expression of TNF-α gene was found in Group M1–3 compared with Group C ($p < 0.05$) (Fig. 3).

Association between histological grading, evaluation of TILs and *IL-6* expression levels in canine mammary carcinomas is reported in Fig. 4. In Fig. 5 the association between histological grading, intratumoral necrosis assessment and *TNF-α* gene expression in canine mammary carcinomas is reported. Unfortunately, there was no statistically significant association in either case ($p > 0.05$).

3.5. Expression of interleukins and miRNAs in low vs. high proliferative CMTs

Canine mammary tumors were divided according to the Ki67 index into low and high proliferating tumors (cut-off 33.3%) to compare the mean values of interleukins (*IL-6, TNF-α*) and the expression levels of miRNAs based on the main immunohistochemical prognostic factor (Table 3).

No significant differences were found in the gene expression of interleukins and miRNAs investigated here between tumors with Ki67 index >33.3% and < 33.3% ($p > 0.05$, Fig. 6).

Table 2
Histological Classification of Canine Mammary Carcinomas, Stromal TILs infiltration and Tumor Necrosis.

Histological Classification	n	Grade of malignancy	Lymphovascular invasion	TILs	Tumor Necrosis (score)
Tubular Carcinoma	1	I		High	2
	1	III	Yes	Low	2
Tubulopapillary Carcinoma	1	I		Low	2
Complex Carcinoma	1	I		Low	1 (Absent)
	1	I		Intermediate	1
	1	I		Intermediate	2
Intraductal Papillary Carcinoma	1	I		Low	3
	1	I		Intermediate	2
Mixed Carcinoma	1	I		Low	2
	1	II		Intermediate	2
Solid Carcinoma	1	II		Low	1 (Absent)
	1	III		Low	2
	1	III		High	2
	1	III		High	2
Inflammatory Carcinoma	1	III	Yes	High	1 (Absent)

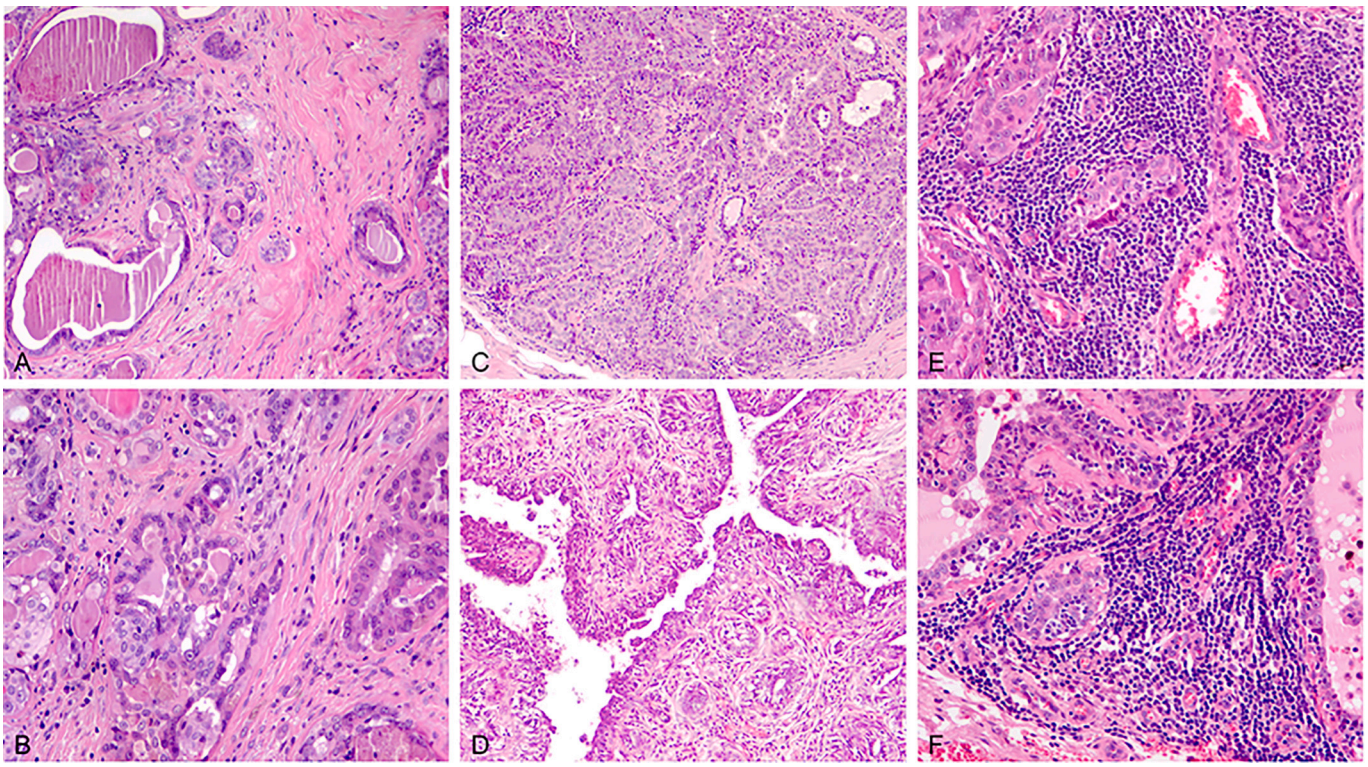


Fig. 1. Stromal TILs infiltration in Canine Mammary Carcinomas. A-B. Low (0–10%) TILs infiltration, Complex Carcinoma, grade I; C. Intermediate (11–40%) stromal TILs infiltration, Solid Carcinoma, grade 3. D. Intermediate (11–40%) TILs infiltration, Complex Carcinoma, grade 1; *E*-F. High (41–90%) stromal TILs infiltration, Solid Carcinoma, grade 3. (A, C, D magnification 100×; B,E,F magnification 200×).

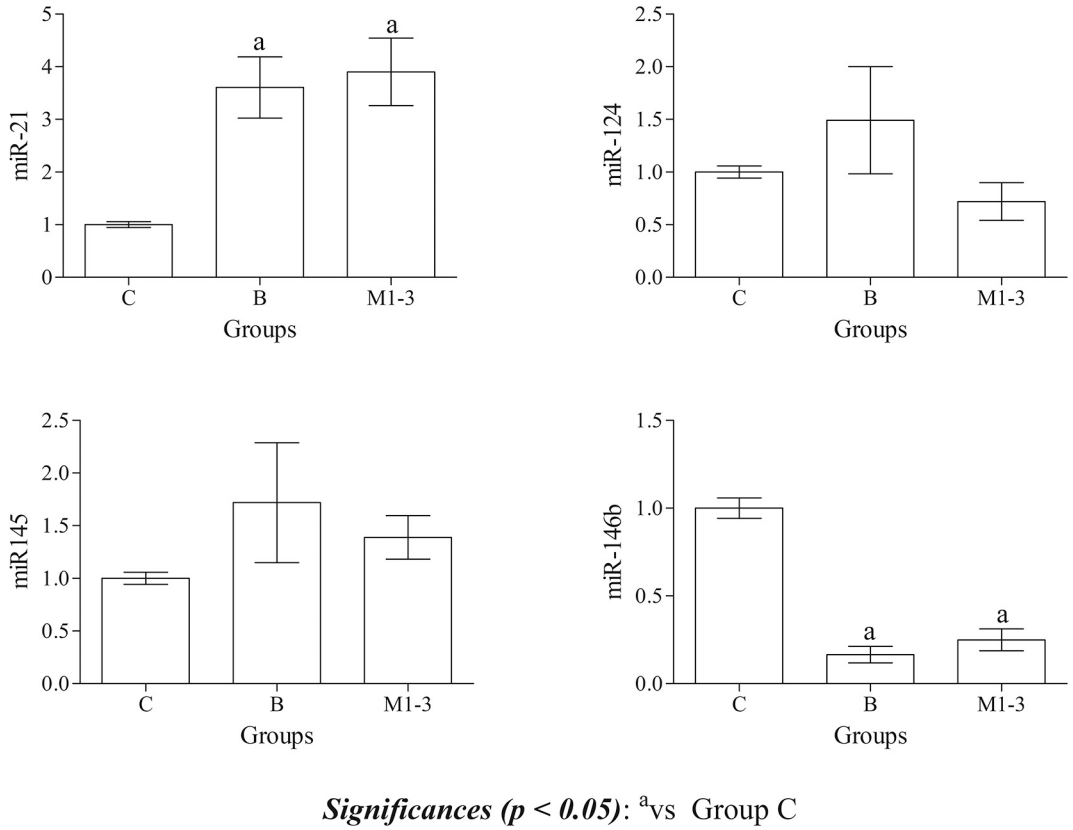
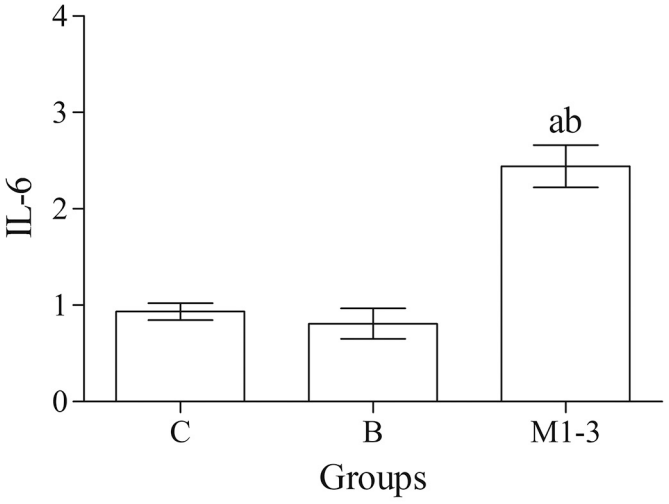
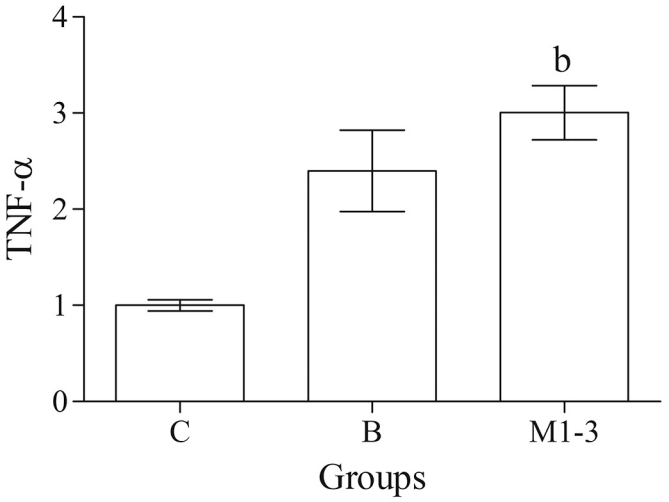


Fig. 2. Expression levels of miRNAs in mammary samples. miR-21 was overexpressed in malignant tumors (M1–3) and benign tumors (B) compared with the control group (C), whereas miR-146b showed a downregulation in malignant tumors (M1–3) and benign tumors (B) than control group (C). No significant difference in the expression levels of miR-124 and miR-145 were observed among groups.





Significances: [a]vs Group B; [b]vs Group C ($p < 0.05$)

Fig. 3. Gene expression of interleukins in canine mammary samples. IL-6 showed an overexpression in malignant tumors (M1–3) compared with the control group (C) and benign tumors (B), whereas, TNF-α was overexpressed in malignant tumors (M1–3) than control group (C).

3.6. Correlation between interleukins and miRNAs expression levels in benign and malignant CMTs with different Ki67 proliferation index

According to the Spearman test results, *IL-6* gene expression showed a significant positive correlation with miR-21, while *IL-6* showed a negative correlation with miR-146b. *TNF-α* gene expression was positively correlated with miR-21 and miR-145 in all malignant tumors (Group M1–3) (Table 4). No significant correlation between interleukins and miRNAs gene expression was observed in benign tumors (Table 4). The Ki67 index showed a significant positive correlation with the gene expression of *IL-6* and miR-21 and a negative correlation with miR-145 and miR-146b in Group M1–3 (Table 4). The Ki67 index of the benign tumors did not correlate with the gene expression of the cytokines and/or miRNAs studied here (Table 4). The gene expression of *IL-6* and miR-21 showed a significant positive correlation with the Ki67 index >33.3%, while the gene expression of miR-145 and miR-146b showed a negative correlation with the Ki67 index >33.3% (Table 5).

The statistical significance of Spearman's test was confirmed by the linear regression model (Figs. 7-9).

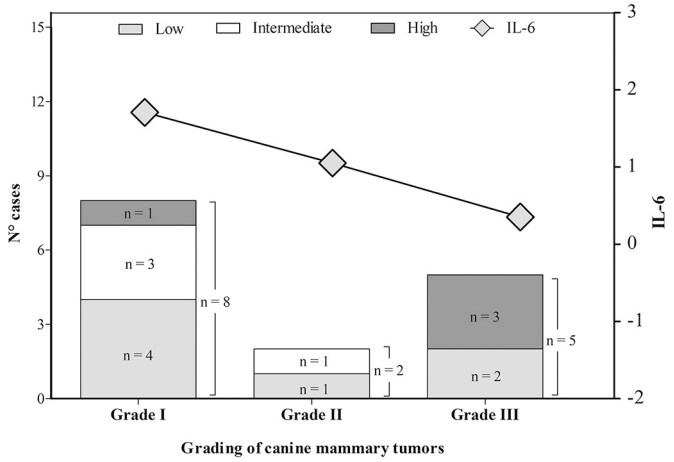


Fig. 4. Histograms showing association between histological grading, TILs and *IL-6* expression.

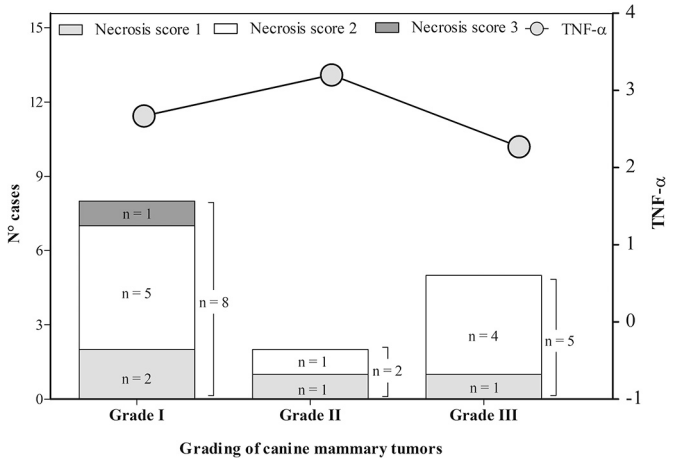


Fig. 5. Histograms showing association between histological grading, intratumoral necrosis assessment and *TNF-α* expression levels.

Table 3
IL-6, *TNF-α* and MiRNAs expression in low and high proliferative canine mammary tumors based on Ki67-index. Data are presented as Mean ± SD. Gene expression levels are presented in terms of fold change normalized to endogenous controls using the formula $2^{-\Delta\Delta Cq}$.

Ki-67 Index	*n*	*IL-6*	*TNF-α*	miR-21	miR-124	miR-145	miR-146b
<33.3% (19.39 ± 7.93)	10	1.31 ± 1.47	2.59 ± 1.66	4.24 ± 2.42	0.88 ± 0.97	1.31 ± 0.71	0.17 ± 0.06
>33.3% (60.43 ± 13.86)	12	0.83 ± 0.57	2.63 ± 1.71	3.48 ± 2.13	1.15 ± 1.21	1.69 ± 1.43	0.24 ± 0.28

4. Discussion

Nowadays, tumor microenvironmental factors in canine mammary cancer are still poorly characterized and, to the Authors' knowledge, this is the first study evaluating the expression of miRNAs predicted to modulate interleukin-mediated inflammation and tumor cell proliferation, along with major interleukin genes. In particular, the RT-qPCR expression levels of selected oncogenic and tumor suppressor miRNAs

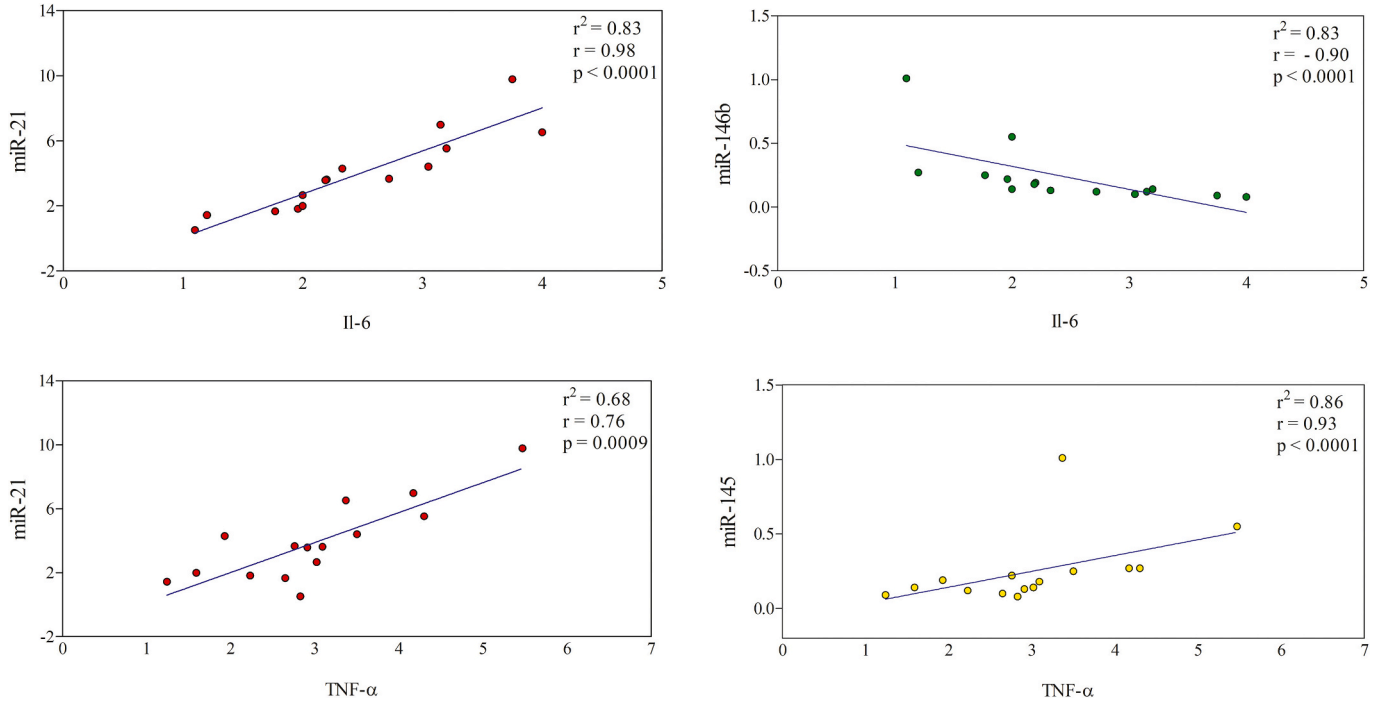


Fig. 6. Expression levels of interleukins (*i.e.* IL-6, TNF-α) and miRNAs (*i.e.* miR-21, miR124, miR145, miR146b) between mammary tumors with Ki67 index >33.3% (high proliferative tumors) and Ki67 index <33.3% (low proliferative tumors).

Table 4
Correlation coefficients (Spearman's *r* and *p* values) between the expression levels of *IL-6* and *TNF-α*, Ki67-index and the expression of miR-21, miR-124, miR-145 and miR-146b in benign (Group B) and malignant tumors (Group M1–3). *P*-values <0.05 were considered statistically significant and highlighted in bold.

		Ki67 index	miR-21	miR-124	miR-145	miR-146b
Group B	***IL-6***	*r* = −0.12 *p* = 0.79	*r* = −0.62 *p* = 0.12	*r* = 0.36 *p* = 0.39	*r* = 0.12 *p* = 0.79	*r* = 0.48 *p* = 0.24
	TNF-α	*r* = −0.21 *p* = 0.62	*r* = −0.26 *p* = 0.54	*r* = 0.67 *p* = 0.08	*r* = 0.40 *p* = 0.33	*r* = 0.5 *p* = 0.22
	Ki67 index		*r* = 0.57 *p* = 0.14	*r* = −0.29 *p* = 0.50	*r* = −0.36 *p* = 0.38	*r* = −0.31 *p* = 0.46
Group M1–3	***IL-6***	***r* = 0.58 *p* = 0.02**	***r* = 0.98 *p* < 0.0001**	*r* = 0.12 *p* = 0.68	*r* = −0.002 *p* = 0.99	***r* = −0.90 *p* < 0.0001**
	TNF-α	*r* = −0.37 *p* = 0.17	***r* = 0.76 *p* = 0.0009**	*r* = 0.01 *p* = 0.96	***r* = 0.93 *p* < 0.0001**	*r* = 0.38 *p* = 0.17
	Ki67 index		***r* = 0.69 *p* = 0.005**	*r* = 0.31 *p* = 0.27	***r* = −0.87 *p* < 0.0001**	***r* = −0.95 *p* < 0.0001**

Table 5
Correlation coefficients (Spearman's r and p values) between Ki67 index < 33.3% (low proliferative tumors) and Ki67 index> 33.3% (high proliferative tumors), *IL-6* and *TNF-α* expression levels and miRNAs (*i.e.* miR-21, miR-124, miR 145 and miR-146b). *P*-values <0.05 were considered statistically significant and highlighted in bold.

	IL-6	*TNF-α*	miR-21	miR-124	miR-145	miR-146b
Ki67 index< 33.3%	*r* = −0.24 *p* = 0.51	*r* = −0.39 *p* = 0.26	*r* = −0.08 *p* = 0.83	*r* = 0.18 *p* = 0.63	*r* = −0.24 *p* = 0.44	*r* = 0.24 *p* = 0.50
Ki67 index > 33.3%	***r* = 0.93 *p* < 0.0001**	*r* = −0.22 *p* = 0.50	***r* = 0.78 *p* = 0.003**	*r* = −0.08 *p* = 0.81	***r* = −0.80 *p* = 0.002**	***r* = −0.77 *p* = 0.004**

(*i.e.* miR-21, miR-124, miR-145 and miR-146b) and pro-inflammatory cytokine genes (*i.e. IL-6* and *TNF-α*) were investigated using FFPE canine mammary tumors. Significant upregulation of the miR-21 oncogene along with significant downregulation of the miR-146b tumor suppressor gene were observed in both malignant and benign CMTs compared with nonneoplastic mammary tissues. Furthermore, *IL-6* gene expression was upregulated in malignant tumors of different histological grade (Group M1–3) compared with benign tumors (Group B) and control cases (Group C), while the expression of *TNF-α* gene was upregulated in Group M1–3 compared with the control group (Group C). These findings appear to reinforce the concept of the close interaction between inflammatory mediators and cancer progression. Of note, miR-21 oncogene was positively correlated with *IL-6* and *TNF-α* gene expression and Ki67 index in malignant mammary tumors, especially in highly proliferating tumors, suggesting a promising prognostic value of this biomarker.

Cancer progression is closely linked to inflammation, which is a key component in orchestrating the development of the TME (Chakraborty et al., 2020). Indeed, tumor-infiltrating inflammatory cells influence cancer growth and resistance to anti-cancer therapy through the secretion of numerous cytokines, which can also be produced by the neoplastic cells themselves, together with growth factors and miRNAs, which represent the main epigenetic mechanism in the modulation of the TME (Bell and Taylor, 2017; Chakraborty et al., 2020; Landskron et al., 2014). Several exosomal and non-exosomal miRNAs have been found to be involved in the regulation of cytokine signaling and cancer-related inflammation, and of note, upregulation or downregulation of

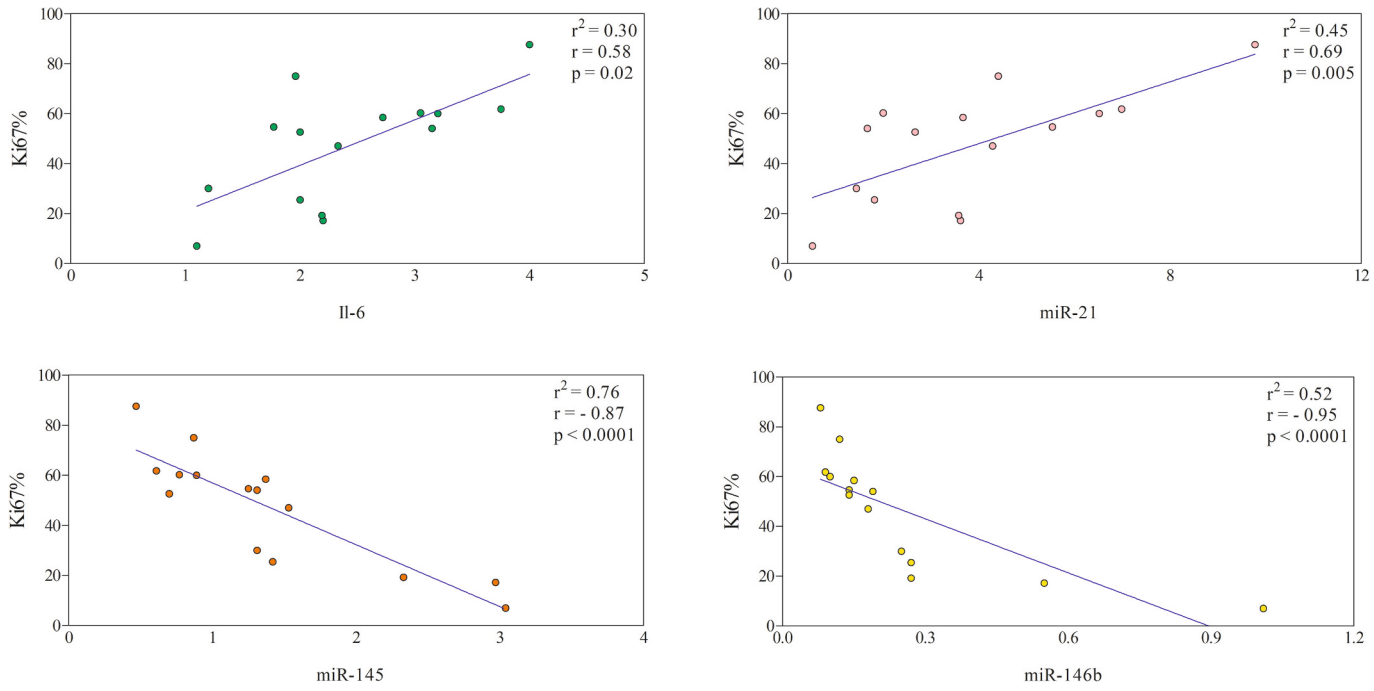


Fig. 7. Linear regression obtained between the expression levels of *IL-6*, miR-21 and miR146b, and, between the expression levels of *TNF-α*, miR-21 and miR-145 in malignant tumors (Group M1–3). *IL-6* expression levels showed a positive correlation with miR-21 and a negative correlation with miR-146b; *TNF-α* expression levels positively correlated with miR-21 and miR-145. The colored circles refer to miRNA expression levels in CMTs.

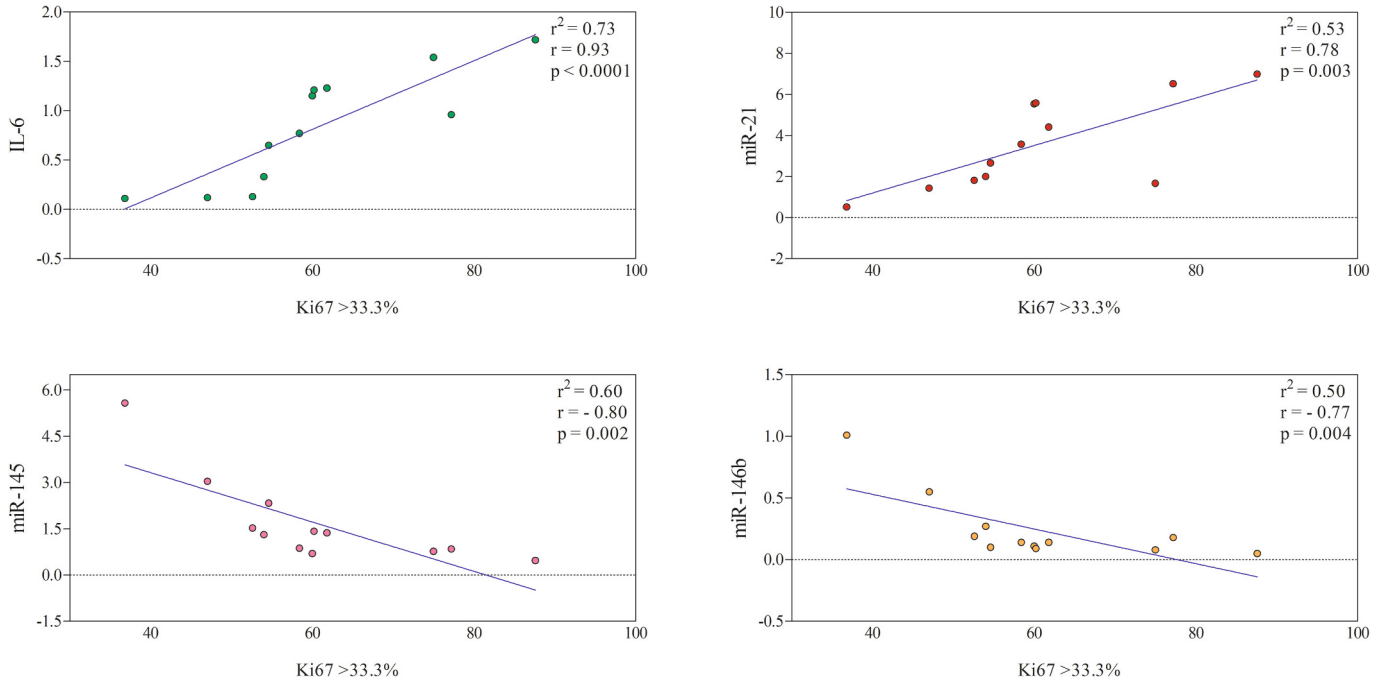


Fig. 8. Linear regression obtained between the Ki67 index and *IL-6*, miR-21, miR-145 and miR-146b expression levels in malignant tumors (Group M1–3). The Ki67 index showed a positive correlation with the expression levels of *IL-6* and miR-21, while it was negatively correlated with the gene expression of miR-145 and miR-146b. The colored circles refer to miRNA expression levels in CMTs.

different miRNAs leads to the development of immune tolerance, potential autoimmunity, hyperinflammatory phenotype and cancer initiation and progression (Anfossi et al., 2018; Chakraborty et al., 2020; Landskron et al., 2014). Therefore, an in-depth knowledge of the complexity of this network of biological factors, together with the characterization of the genes involved, can represent a useful tool to better understand the molecular basis of cancer.

It is well known that during tumor progression, changes in the TME induce a switch in innate immune cells towards a pro-tumorigenic function and actively contribute to immune tolerance, preventing tumor rejection by the immune system (Carvalho et al., 2014). Tumor-infiltrating lymphocytes (TILs) are crucial players in shaping the immune response against cancer, especially T lymphocytes (Bujak et al., 2020). Furthermore, TILs in the TME produce both type 1 (*e.g.* TNF-a)

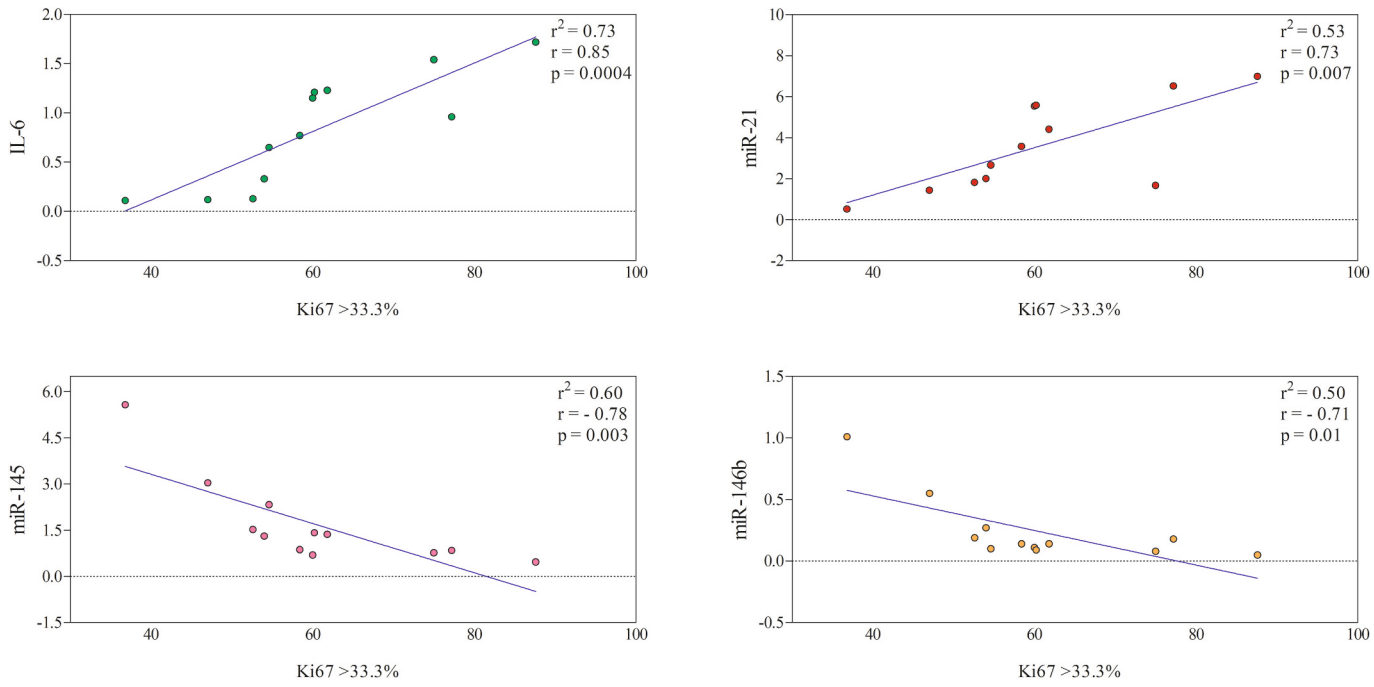


Fig. 9. Linear regression obtained between the Ki67 index >33.3% (high proliferative tumors) and the expression levels of *IL-6*, miR-21, miR-145 and miR-146b. The Ki67 index >33.3% showed a significant positive correlation with *IL-6* and miR-21 expression levels, while it was negatively correlated with miR-145 and miR-146b expression levels. The colored circles refer to miRNA expression levels in CMTs.

and type 2 cytokines (*e.g.* IL-6) and can encourage tumor growth by promoting angiogenesis and lymphangiogenesis (Kim et al., 2010). Although the association between TILs and TME is well known, the results obtained in the present study do not support the association between *IL-6*, *TNF-α* and investigated miRNAs with TILs or tumor necrosis scores. Since the data may be biased by the number of samples and histotypes used, further studies will be needed, investigating a larger number of cases and a larger panel of interleukins since other pro-inflammatory cytokines can be secreted within the TME to better understand the cellular and molecular pathways involved in cancer-related inflammation. In our study, although most carcinomas showed a low infiltration of TILs, high TILs infiltration was observed in high-grade carcinomas (n = 2 solid carcinomas, grade III; Inflammatory carcinoma, grade III), whereas, both *IL-6* and *TNF-α* genes were upregulated in all carcinomas compared with benign tumors and nonneoplastic mammary tissues.

Among cytokine, IL-6 is implicated as a key cytokine linking inflammation with carcinogenesis and is overexpressed in several cancer types (Zarogoulidis et al., 2013), Indeed, IL-6 is recognized as having a strong pro-carcinogenic activity due to its role as an amplifier of inflammation and its effects on the proliferation, survival and invasion of cancer cells (Changkija and Konwar, 2012). This cytokine is one of the activation signals of the nuclear transcription factor kappa B (NF-κB), an inflammation-amplifying circuit that potentiates carcinogenesis by promoting cell differentiation and subsequent metastasis (Germano et al., 2008). Elevated NF-κB expression positively modulates IL-6 and TNF-α expression levels in mammary tumors (Martins et al., 2016). At the same time, TNF-α promotes cell proliferation and indirectly inhibits apoptosis, through the induction of NF-κB, and acts as a potent inducer of pro-inflammatory cytokines, such as IL-1 and IL-6 (Le Bitoux and Stamenkovic, 2008; Martins et al., 2016; Soria et al., 2011; Tang et al., 2002). In this study, *IL-6* gene expression showed a strong positive correlation with the expression of the miR-21 oncogene and a negative correlation with the miR-146b tumor suppressor gene in mammary carcinomas. Furthermore, a strong positive correlation was found between the gene expression of *TNF-α*, miR-21 and miR-145 only in mammary carcinomas. Noteworthy, when exosomal miR-21 attaches with Toll-like receptor 7/8 (TLR7/8) on macrophages, it helps promote IL-6 secretion, leading to enhancement of proinflammatory response, while miR-146b inhibits the NF-κB-mediated production of IL-6 and IL-6/STAT3-driven breast cancer cell migration and invasion (Anfossi et al., 2018; Chakraborty et al., 2020; Xiang et al., 2014). Indeed, miR-146b inhibits NF-κB-dependent IL-6 production, subsequent STAT3 activation, and IL-6/STAT3-driven migration and invasion in breast cancer cells, thus establishing a negative feedback loop (Xiang et al., 2014). Regarding miR-145, it is assumed to be a tumor suppressor gene and appears to be downregulated in several cancer types (Sachdeva et al., 2009), including triple-negative breast cancer (TNBC) tissue and MDA-MB-231 cell line, in which miR-145 overexpression is induced by TNF-α and results in cell death and apoptosis (Zheng et al., 2016).

The positive correlation between miR-21 and *TNF-α* obtained in our study may suggest a key functional role of this miRNA on inflammation also in the canine mammary TME, where miR-21 may act to promote NF-κB-mediated production of both IL-6 and TNF-α, leading to the enhancement of the pro-inflammatory response (Chakraborty et al., 2020). Moreover, miR-21 serves as a key regulator of tumor cell proliferation and has been suggested as a promising biomarker in metastases detection (Zhang et al., 2016).

In the present study, the highest level of miR-21 gene expression was observed in malignant CMTs with histological evidence of lymphovascular invasion (*i.e.* inflammatory carcinoma, grade III; tubular carcinoma, grade III), although no metastases have been observed in the draining lymph node. A strong correlation was found in malignant CMTs between miR-21 and *IL-6* expression levels and tumor cell proliferative index (Ki67 index), as main prognostic immunohistochemical factor. In particular, the Ki67 index was positively correlated with the expression of miR-21 and *IL-6* in high proliferating tumors (Ki67 > 33.3%). Conversely, a negative correlation was found between the cell proliferation index (Ki67 index) and the tumor suppressor genes miR-145 and miR146b. It is well known that malignant CMTs with increased tumor cell proliferation, as measured by the Ki67 index, are associated with a poor prognosis (Sarli et al., 2002; Zuccari et al., 2004). Thus, the correlation results suggest a central role of miR-21 and *IL-6* in the malignant phenotype of mammary epithelial cells and suggest that miR-21 is a

promising biomarker with prognostic value.

5. Conclusions

Gathered results reinforce the idea of the central functional role of cytokines and inflammatory cells within the tumor microenvironment and suggest the central role of miR-21 and miR-146b in mammary cancer pathophysiology and progression. Since the upregulation of miR-21 oncogene closely reflects gene overexpression of the interleukins *IL-6* and *TNF-α* and a high tumor proliferation index, this miRNA may be considered a promising biomarker with prognostic value worthy of future investigations. A richer panel of interleukins and miRNAs should be investigated to characterize the molecular patterns and relationships between inflammatory cells and tumor cells involved in cancer biology. Indeed, only a clear understanding of the role of miRNAs in cytokine signaling pathways and cancer-related inflammation can help develop promising anti-cancer therapeutic agents, which could lead to better treatment protocols in the near future in canine patients and which could benefit future human breast cancer clinical trials.

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Declaration of competing interest

The authors declare no conflict of interest.

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